

High Frequency Direct Organogenesis Protocol for Mass Multiplication of *Centrosema pubescens* Benth

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Abstract

This study was aimed to develop a suitable protocol for rapid and mass propagation of *Centrosema pubescens* Benth. Fresh seeds from field grown plants were aseptically cultured on full and half-strength MS media in combination with or without PGRs (plant growth regulators). Cent percent seed germination was achieved on half-strength MS medium without any PGRs and MS medium in combination with 1.0 mg/l BAP. The shoot apices and nodal segments from *in vitro* grown seedlings were aseptically cultured for direct organogenesis. The MS medium supplemented with 2.0 mg/l BAP + 1.0 mg/l IAA produced maximum number of multiple shoot buds (4.44 ± 0.12) per shoot apices segment. On the other hand, 93% of nodal segments gave response for induction of multiple shoot buds when cultured them on MS medium supplemented with 2.0 mg/l BAP + 1.0 mg/l NAA. The highest percentage of leaf segments (86%) produced brownish friable callus on MS + 1.0 mg/l 2, 4-D + 0.5 mg/l BAP. It was reported that brownish friable callus did not undergo any kind of differentiation. Maximum root induction was 5.20 ± 0.10 per elongated shoot and the highest average length 3.07 ± 0.26 cm was observed on half-strength MS medium fortified with 1.0 mg/l IAA. The strong and stout rooted plantlets were successfully hardened with 78% of survival rate. Further research could be initiated to produce secondary metabolites from the friable callus of this plant species to fulfill the demands of the pharmaceutical industries.

Introduction

Centrosema pubescens Benth. commonly known as “Centro” or “Butterfly Pea” belongs to Fabaceae family is characterized by herbaceous, twinning, and climbing stem. It is native to tropical regions. In Bangladesh, it may be seen in tea estates, roadside, and fallow lands in some parts of Sylhet, Chattogram, and Mymensingh regions. This plant is most well-known for treating skin infections in humans (Chukwuma et al. 2015). In Laos, the sap extracted from the seed of the *C. pubescens* is used as anesthetic agent for scorpion

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and snake bites (Ekpo et al. 2011). It also prescribed for women for womb cleansing (Ariwaodo et al. 2012). The species is used as food and fodder (Chima et al. 2013). Leaves of *C. pubescens* contain dry matter 23.3%, crude fibre 30.8%, crude extract 3.9%, crude protein 21% and total digestible nutrient 4.9%. Being a leguminous plant, it can be used as livestock feed additive (Uzuomfor 2015). Several studies had already been conducted regarding the biological activities of *Centrosema*, such as cytotoxic (Interino et al. 2023) and wound healing properties (Ekpo et al. 2011). Oluwatosin (2013) reported that the antagonistic properties of *C. pubescens* against 15 bacterial isolates. The methanolic leaf extract of *C. pubescens* shows strong antioxidant and antimicrobial activity (Murugan 2020). In addition, this species increases soil fertility and soil organic matter through leaf litter. It is rich in protein and contributes to soil nitrogen and carbon. The symbiotic interaction between rhizobia and *Centrosema* is one of the dependable sources that provide nutrients to plants, increases soil fertility, facilitates plant growth and restores degraded ecosystem. Legume plant helps to nitrogen fixation and agroecological management allows nutrient recycling (Chavarria et al. 2018).

Despite its promising application, over-harvesting, rapid urbanization and industrialization have led to the depletion of plant resources, placing it at risk of extinction. Besides seed dormancy, rare seed set production, loss of viability, and endophytic bacterial and fungal contamination in explants are some of the major limitations in the conventional propagation. Micropropagation, a technique involving the vegetative multiplication of plants from viable and regenerative cells in a controlled, sterile environment using appropriate culture media, has proven effective for medicinal plant production (Zhou and Wu 2006). Furthermore, it facilitates the rapid distribution of elite or improved genotypes across different geographical regions, thus supporting agricultural and pharmaceutical industries (Thorpe 2007). The research work was initiated to develop an efficient and reproducible *in vitro* regeneration protocol of *Centrosema pubescens* for both conservation and medicinal utilization purposes. The present findings will be useful for further researches of this species.

Materials and Methods

Seeds of fresh fruits were obtained from the Chittagong University campus, Bangladesh. Collected seeds were stored under dry conditions at room temperature. For the initiation of aseptic culture, the collected seeds were washed thoroughly under running tap water and surface sterilized with 1% savlon and liquid soap for 5-10 min with constant shaking. The seeds were then washed 3-4 times with distilled water for complete removal of detergent and taken them under laminar air flow cabinet and transferred them to 500 ml sterilized conical flask. After rinsing with 70% (v/v) ethanol for less than 60 sec, and rinsed three times in sterile distilled water. Afterwards, they were immersed in 0.1% HgCl₂ solution for different duration of time. To remove every trace of the sterilant materials were washed 4-5 times with sterile distilled water. Then, the sterilized seed were cultured in prepared culture vessels.

The MS basal medium supplemented with 30 g/l sucrose supplemented with cytokinins (BAP and Kn) and auxins (IAA, IBA and NAA) were used for different experiments. The pH of the medium was adjusted to 5.80 before adding solidifying agent (12 g/l agar). The medium was autoclaved at 15 psi pressure and 121°C for 20 min. The autoclaved medium was allowed to cool in a sterile environment. Then cultures were maintained in a growth chamber at 25 ± 2°C and the light intensity was 2000-3000 lux with cool white fluorescent lamps under 16 hrs photoperiod.

For shoot induction, shoot apices (1-1.5 cm) were taken from *in vitro* germinated seedlings and cultured on 0.8% agar solidified MS basal medium fortified with different concentration of PGRs. These were sub-cultured on the same media for further response at an interval of 15-20 days and culture vessels were maintained in the culture room in the same light and temperature conditions. The percentage of shoot tips that developed shoots, the number of shoots per explant, and the height of micro-shoot were recorded after 5 weeks. For callus induction, explants were cultured on the MS medium containing 2, 4-D singly and in combination with Kn and BAP. On the other hand, micro-shoots that responded well on *in vitro* shoot tip cultures were transferred to a shoot multiplication medium. After five weeks, the multiplied shoots were separated and sub-cultured on the elongation medium under sterile conditions. Lengths of shoots were recorded after four weeks of initial culture.

To develop complete plantlets, elongated shoot buds measuring around 2-3 cm were separated and placed in rooting media. Initially, half-strength MS medium without any PGRs was used. Later, for better rooting results, half-strength MS medium was enriched with different concentrations and combinations of auxins (IBA, IAA, and NAA). The number of roots, root length, and shoot height were measured after five weeks of culture initiation.

For acclimatization of *in vitro* developed complete plantlets in the outside environment, the culture vessels with well profuse rooted plantlets were taken out of the culture room and kept in ordinary room temperature with gradual increasing the time up to 5 days. Finally, the plantlets were taken out from the culture vessels and washed with tap water for complete removal of agar attached to the roots. Then, the plantlets were planted in small plastic pots containing garden soil and compost in the ratio of 1 : 1 and kept in room temperature for 3-5 days. After gradual acclimatization, the pots with *in vitro* developed plantlets were finally transferred to the outside natural environment. The percentage of survived plants was recorded after two months. Each treatment was repeated thrice. The graphs and mean values of parameters were compared by analysis of variance using SPSS (Ver. 26) software.

Results and Discussion

After three weeks of culture initiation, the cent per cent aseptic seed germination rate of *C. pubescens* was recorded where healthy green seedlings were found in half-strength MS medium without any PGRs and full MS in combination with 1.0 mg/l BAP (Fig. 1). Shoot

apices, and nodal segments obtained from *in vitro*-grown seedlings were further cultured on 0.8% w/v agar-solidified MS medium supplemented with cytokinins (BAP and Kn) in combination with auxins (NAA, IAA, and 2,4-D). Among the explants, both the shoot apices and nodal segment exhibited direct organogenesis, with the nodal segments showing a more favorable response compared to the shoot apices. These explants directly differentiated into shoot buds when cultured on MS medium enriched with various growth regulator combinations.

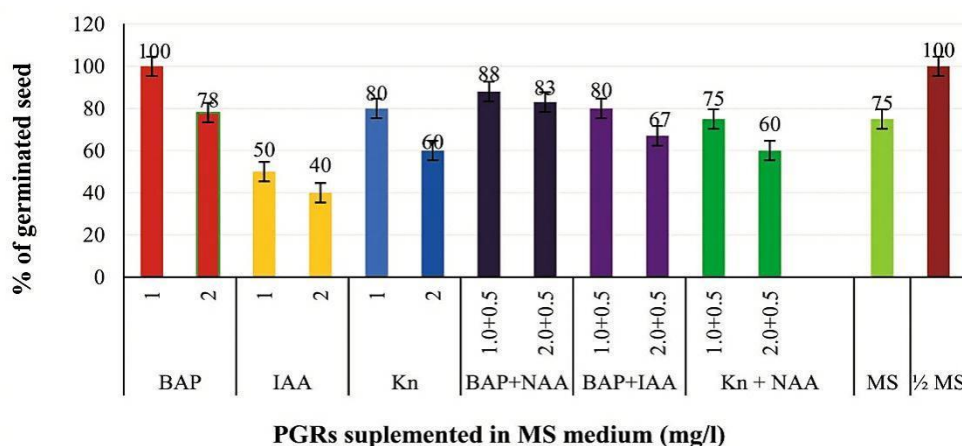


Fig. 1. *In vitro* seed germination experiment of *C. pubescens*.

A significant variation in shoot bud induction was observed based on the concentrations of BAP used alone or in combination with NAA, IAA, Kn and Kn combined with NAA and IAA formation of shoot buds from nodal and shoot apices explants. The percentage of shoot apices showing proliferation varied from 45 to 89%. The highest number (89%) of shoot apices produced maximum average number of shoot buds (4.44 ± 0.12) per explant in MS medium that supplemented with 2.0 mg/l BAP + 1.0 mg/l IAA (Table 1). The shoot buds were induced within 12-15 days of culture. The lowest (45%) shoot apices showed proliferation when cultured them on MS medium that contained 1.0 mg/l Kn + 0.5 mg/l NAA within 25-30 days of culture. The findings highlighted the efficacy of cytokinin (BAP) and auxin (IAA) combination initiated the multiple shoot formation. Previous studies on leguminous plants such as *Clitoria ternatea* and *Centrosema pascuorum* reported similar findings where BAP in combination with a low concentration of auxin enhanced shoot bud induction (Murthy et al. 2012, Rout et al. 2000) and *Nephelium lappaceum* (Putri et al. 2022).

On the other hand, 93% of nodal segments gave response for induction of multiple shoot buds when cultured them on MS medium supplemented with 2.0 mg/l BAP + 1.0 mg/l NAA and this medium also produced maximum number of shoot buds (2.27 ± 0.19) per nodal segment. Whereas, the lowest number of nodal explants (60%) produced shoot buds in the MS medium containing 2.0 mg/l Kn + 1.0 mg/l IAA with minimum number of shoot buds (1.13 ± 0.33) per nodal segment (Table 1).

Table 1. Effects of different concentrations and combinations of PGRs on induction of shoot buds from shoot apices and nodal segment explant of *C. pubescens*.

PGRs concentration in MS medium (mg/l)				Shoot apices		Nodal segments	
BAP + NAA	BAP + IAA	Kn + NAA	Kn + IAA	Percentage* of explants showed proliferation (Mean ± SE)	Average* no. of shoot buds per culture (Mean ± SE)	Percentage* of explants gave response (Mean ± SE)	Average* no. of shoot buds per culture (Mean ± SE)
1.0 + 0.5	-	-	-	86.29 ± 0.01 ^a	4.29 ± 0.11 ^c	80.29 ± 0.01 ^a	1.93 ± 0.20 ^a
1.0 + 1.0	-	-	-	83.24 ± 0.01 ^a	3.33 ± 0.23 ^d	73.24 ± 0.01 ^a	1.35 ± 0.32 ^d
2.0 + 1.0	-	-	-	88.04 ± 0.09 ^c	4.30 ± 0.14 ^a	93.04 ± 0.09 ^c	2.27 ± 0.19 ^a
-	1.0 + 0.5	-	-	86.26 ± 0.03 ^a	3.43 ± 0.31 ^a	80.26 ± 0.03 ^d	2.00 ± 0.15 ^a
-	1.0 + 1.0	-	-	75.31 ± 0.02 ^c	2.25 ± 0.20 ^a	66.31 ± 0.02 ^a	1.40 ± 0.28 ^d
-	2.0 + 1.0	-	-	89.00 ± 0.01 ^a	4.44 ± 0.12 ^c	73.00 ± 0.01 ^a	1.56 ± 0.26 ^a
-	-	1.0 + 0.5	-	45.01 ± 0.03 ^a	1.33 ± 0.26 ^a	80.01 ± 0.03 ^a	1.85 ± 0.35 ^a
-	-	1.0 + 1.0	-	50.31 ± 0.02 ^d	1.50 ± 0.22 ^a	67.31 ± 0.02 ^b	1.73 ± 0.33 ^c
-	-	2.0 + 1.0	-	67.27 ± 0.03 ^a	2.00 ± 0.17 ^a	87.27 ± 0.03 ^a	1.67 ± 0.12 ^a
-	-	-	1.0 + 0.5	71.25 ± 0.02 ^a	2.14 ± 0.28 ^a	73.25 ± 0.02 ^a	1.40 ± 0.22 ^b
-	-	-	1.0 + 1.0	86.29 ± 0.04 ^b	3.43 ± 0.29 ^a	80.29 ± 0.04 ^c	1.80 ± 0.16 ^a
-	-	-	2.0 + 1.0	57.27 ± 0.03 ^a	1.48 ± 0.10 ^b	60.27 ± 0.03 ^a	1.13 ± 0.33 ^c

*Values are the means of five replicates of 15 explants. All test values with different superscripts in the same column are significantly different at $p \leq 0.05$.

From the overall results for induction of shoot buds in both explants of *C. pubescens* underwent direct organogenesis but the nodal segments were better than shoot apices. Relatively higher amount of cytokinin in combination with lower amount of auxin promoted direct organogenesis which was better in combination of BAP and NAA than that of any other concentration and combination of PGRs. In addition, BAP was reported to be more stable in tissue culture when compared to other cytokinins due to its slower rate of metabolism. The importance of plant growth regulators on shoot propagation was highlighted in various studies. Particularly, the synergistic effect of BAP with NAA has been shown in many other medicinal plants, *Centella asiatica* (Hossain et al. 2000), *Catharanthus roseus* (Islam et al. 2001), *Aloe vera* (Das and Rout 2003), *Boerhaavia diffusa* (Roy 2008), *Stemona tuberosa* (Biswas et al. 2011), *Plectranthus amboinicus* (Rahman et al. 2015), *Coccinia grandis* (Kashem and Rahman 2018), *Leucas biflora* (Paul and Biswas 2024), *Spilanthes acmella* (Sana and Rani 2025).

The leaf segments produced brownish friable callus when cultured on MS medium fortified with different concentrations of auxin and cytokinin. The highest number of leaf segments (86%) produced callus tissue on MS medium containing 1.0 mg/l 2, 4-D + 0.5 mg/l BAP (Fig. 2). The nature of callus in all the media compositions was brownish friable. However, minimum callus induction frequency (65%) was noted in MS medium containing 1.0 mg/l 2, 4-D alone. Similar proliferation of callus tissue from leaf segments on MS medium containing 2,4-D and BAP was also noted in other medicinal plants

including *Mirabilis jalapa* (Ling et al. 2009), *Momordica charantia* (Munsur et al. 2013), *Kalanchoe pinnata* (Santos et al. 2014), *Calotropis gigantea* (Muthiah et al. 2023) and *Cynanchum callialatum* (Kashem et al. 2025). In order to promote differentiation, the calli produced from leaf explants of *C. pubescens* were further grown on a broad spectrum of PGRs supplemented MS medium. It was highly noticed that the brownish friable callus does not undergo any kind of differentiation and finally died.

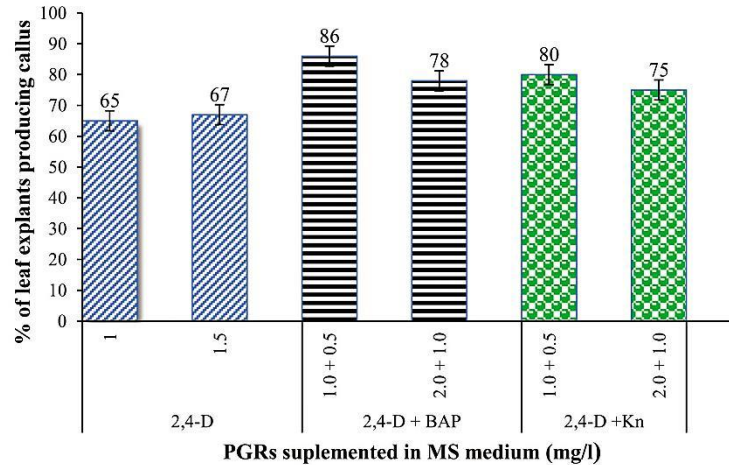


Fig. 2. Effects of 2, 4-D individually and in combination with BAP and Kn on induction of callus from leaf segment explant of *C. pubescens*.

Table 2. Effects of BAP and Kn individually and in combination with NAA and IAA on elongation of directly produced shoot buds of *C. pubescens*.

PGRs supplement in the media (mg/l)		Average* initial length (cm) of individual shoot bud (mean \pm SE)	Average* length (cm) of individual shoot buds after 30d of culture (mean \pm SE)	Average* increase in length(cm) of individual shoot buds after 30d of culture (mean \pm SE)
BAP	Kn			
1.0	-	1.20 \pm 0.12 ^c	3.60 \pm 0.30 ^a	2.40 \pm 0.16 ^c
2.0	-	1.13 \pm 0.24 ^c	3.40 \pm 0.20 ^c	2.27 \pm 0.25 ^a
-	1.0	1.20 \pm 0.40 ^d	3.60 \pm 0.18 ^b	2.40 \pm 0.34 ^c
-	2.0	1.00 \pm 0.32 ^c	3.25 \pm 0.25 ^c	2.15 \pm 0.21 ^d
-	-	1.0 + 0.5	4.20 \pm 0.34 ^b	2.80 \pm 0.26 ^c
-	-	2.0 + 1.0	4.93 \pm 0.17 ^c	3.33 \pm 0.40 ^b
-	-	-	1.0 + 0.5	4.60 \pm 0.29 ^d
-	-	-	2.0 + 1.0	3.07 \pm 0.32 ^a
-	-	-	-	4.20 \pm 0.18 ^c
-	-	-	1.0 + 0.5	2.80 \pm 0.28 ^c
-	-	-	2.0 + 1.0	2.13 \pm 0.11 ^c
-	-	-	-	3.20 \pm 0.20 ^a
-	-	-	-	3.40 \pm 0.40 ^c
-	-	-	-	2.27 \pm 0.16 ^c
-	-	-	-	1.13 \pm 0.24 ^c
-	-	-	-	3.33 \pm 0.28 ^c
-	-	-	-	2.20 \pm 0.30 ^d
-	-	-	-	1.13 \pm 0.24 ^c
-	-	-	-	4.33 \pm 0.33 ^a
-	-	-	-	2.80 \pm 0.35 ^b

d = days. *Values are the means of five replicates with 15 explants. All test values with different superscripts in the same column are significantly different at $p \leq 0.05$.

For promoting rapid elongation, the *in vitro* grown shoot buds of *C. pubescens* that developed directly from shoot apices and nodal segments were further cultured on MS medium supplemented with different concentrations and combinations of PGRs. The highest increase of shoot buds (3.33 ± 0.40 cm) was recorded in MS medium containing 2.0 mg/l BAP + 1.0 mg/l NAA and the minimum elongation (2.13 ± 0.11 cm) was observed on 1.0 mg/l Kn + 0.5 mg/l NAA (Table 2).

The results regarding the elongation of shoot bud indicate that the combination of BAP and NAA was more effective in promoting elongation. Similarly, the effect of BAP in combination with NAA has been noticed in many other medicinal plant species such as *Vitex negundo* (Hiregoudar et al. 2006), *Gynura procumbens* (Keng et al. 2009), *Physalis peruviana* (Guney et al. 2016) and *Physalis minima* (Nahar et al. 2025).

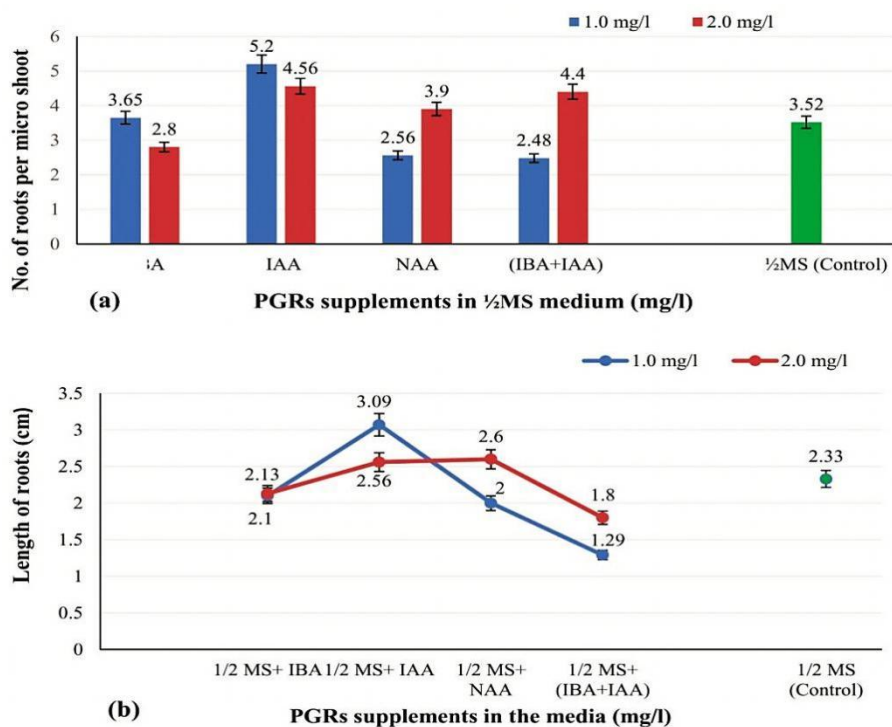


Fig. 3(a-b). Effects of 1/2MS with different auxins on root development of *C. pubescens*: (a) number of roots and, (b) length of roots.

After establishment of well profuse rooted plantlets the *in vitro* raised complete seedlings with healthy root system were transferred to outside environment and planted in earthen pot through successive phase of acclimatization (Fig. 4F). Hardening of *in vitro* grown plantlets for adjustment to natural environment is a difficult aspect of plant tissue culture. These plantlets were developed through *in vitro* culture technique were at last transferred to earthen pots in outside environment. Ultimately 78% of the plantlets survived and established showing normal growth.

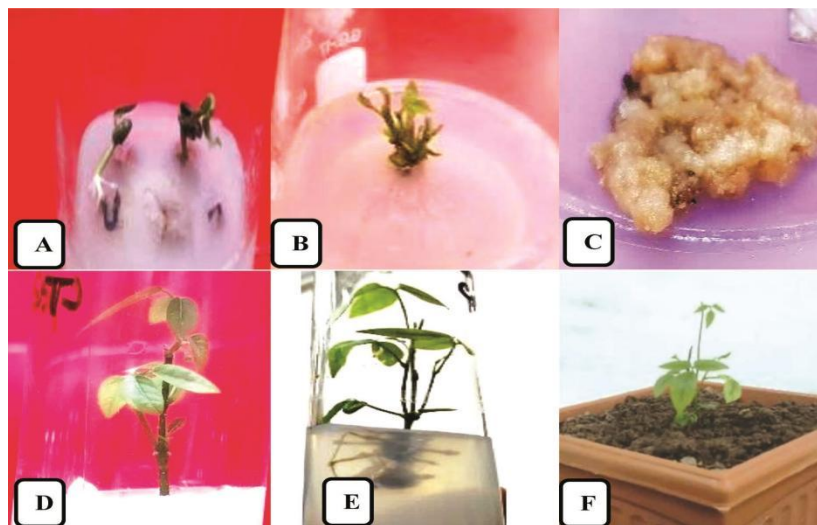


Fig. 4(A-F). *In vitro* propagation of *Centrosema pubescens* through direct organogenesis. (A) Aseptic seed germination, (B) Shoot initiation on MS medium containing 2.0 mg/l BAP combined with 1.0 mg/l IAA, (C) Initiation of brownish friable callus in leaf segments, (D) Maximum elongation of shoot buds on MS + 2.0 mg/l BAP and 1.0 mg/l NAA, (E) Highest number of roots per micro shoot in half-strength MS + 1.0 mg/l IAA, and (F) establishment of *in vitro* raised plantlets in outside environment.

The present investigation successfully established effective *in vitro* protocols for regeneration and rapid propagation of the selected medicinal plant species of *Centrosema pubescens*. Thus, it could be said that the optimized protocol developed in this investigation will aid in the sustainable propagation without any seasonal constraint, conservation, and potential pharmaceutical use of this important medicinal plant species. Further research could be initiated to isolate the phytochemicals from this valuable species. The recorded callus can be used for production of secondary metabolites in pharmaceuticals industry. Despite the promising results, further research is recommended to examine the genetic fidelity of the regenerated plants using molecular markers, assess their secondary metabolite content compared to wild plants, and evaluate their agronomic performance under field conditions. Such studies will strengthen the practical applications of the developed micropropagation systems and support the conservation and commercial cultivation of these valuable species.

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